Drug susceptibility distributions of Mycobacterium chimaera and other non-tuberculous mycobacteria

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Abstract: Recent outbreaks of cardiac surgery-associated Mycobacterium chimaera infections have highlighted the importance of species differentiation within the Mycobacterium avium complex and pointed to a lack of antibiotic susceptibility data for M. chimaera Using the MGIT 960/EpiCenter TB eXiST platform, we have determined antibiotic susceptibility patterns of 48 clinical M. chimaera isolates and 139 other non-tuberculous mycobacteria including 119 members of the M. avium complex and 20 Mycobacterium kansasii towards clofazimine and other drugs used to treat infections with slowly growing nontuberculous mycobacteria (NTM). MIC50, MIC90 and tentative epidemiological cutoff (ECOFF) values for clofazimine were 0.5 mg/L, 1 mg/L and 2 mg/L for M. chimaera. Comparable values were observed for other M. avium complex members, lower MIC50 (0.25 mg/L), MIC90 (0.5 mg/L) and ECOFF (1 mg/L) values were found for M. kansasii Susceptibility to clarithromycin, ethambutol, rifampin, rifabutin, amikacin, moxifloxacin and linezolid was in general similar for M. chimaera and other members of the M. avium complex but increased for M. kansasii The herein determined MIC distributions, MIC90 and ECOFF values of clofazimine for M. chimaera and other NTM provide the basis for the definition of clinical breakpoints. Further studies are needed to establish correlation of in vitro susceptibility and clinical outcome.

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Drug susceptibility distributions of *Mycobacterium chimaera* and other non-tuberculous mycobacteria

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Key words: *Mycobacterium chimaera*, *Mycobacterium avium* complex, drug susceptibility testing, clofazimine, resistance

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ABSTRACT

Recent outbreaks of cardiac surgery-associated *Mycobacterium chimaera* infections have highlighted the importance of species differentiation within the *Mycobacterium avium* complex and pointed to a lack of antibiotic susceptibility data for *M. chimaera*. Using the MGIT 960/EpiCenter TB eXiST platform, we have determined antibiotic susceptibility patterns of 48 clinical *M. chimaera* isolates and 139 other non-tuberculous mycobacteria including 119 members of the *M. avium* complex and 20 *Mycobacterium kansasii* towards clofazimine and other drugs used to treat infections with slowly growing nontuberculous mycobacteria (NTM). MIC_{50}, MIC_{90} and tentative epidemiological cutoff (ECOFF) values for clofazimine were 0.5 mg/L, 1 mg/L and 2 mg/L for *M. chimaera.* Comparable values were observed for other *M. avium* complex members, lower MIC_{50} (≤0.25 mg/L), MIC_{90} (0.5 mg/L) and ECOFF (1 mg/L) values were found for *M. kansasii.* Susceptibility to clarithromycin, ethambutol, rifampin, rifabutin, amikacin, moxifloxacin and linezolid was in general similar for *M. chimaera* and other members of the *M. avium* complex but increased for *M. kansasii.* The herein determined MIC distributions, MIC_{90} and ECOFF values of clofazimine for *M. chimaera* and other NTM provide the basis for the definition of clinical breakpoints. Further studies are needed to establish correlation of *in vitro* susceptibility and clinical outcome.

47 **INTRODUCTION**

48

49 *Mycobacterium chimaera* is a slowly growing non-tuberculous mycobacterium (NTM),
50 which was established in 2004 as a new species within the *Mycobacterium avium*
51 complex (1). In the past, the number of infections with *M. chimaera* was
52 underestimated as commercial mycobacterial identification systems such as line
53 probe assays failed to identify *M. chimaera* to species level and classified
54 *M. chimaera* as *M. avium* complex, *M. avium* or *Mycobacterium intracellulare* (1, 2).
55 *M. chimaera* is differentiated from other members of the *M. avium* complex by a
56 unique 16S rRNA gene sequence and internal transcribed spacer (ITS) region (1).
57 Recently, a global outbreak of cardiac surgery-associated *M. chimaera* infections
58 highlighted the importance of species identification within the *M. avium* complex (3,
59 4). The outbreak was linked to contaminated water reservoirs of heater-cooler
60 devices that spread *M. chimaera* by aerosols during open chest surgery (5, 6).
61 Severe, disseminated *M. chimaera* infections with a high case fatality rate were
62 observed (7).

63

64 Due to the limited ability of commercial identification methods to adequately identify
65 *M. chimaera,* few studies have reported drug susceptibility data on *M. chimaera.*
66 Recent studies analyzed antimicrobial susceptibility of *M. chimaera* using a
67 commercial microdilution system, the SLOWMYCO Sensititre™ panel from Trek
68 Diagnostic Systems, and reported similar susceptibility patterns for *M. chimaera* as
69 for other members of the *M. avium* complex (8-11). Recommended treatment options
70 for disseminated *M. chimaera* infections include combination therapy with
71 macrolides, rifamycins, ethambutol, amikacin and clofazimine (7). Clofazimine is not
72 yet included in the SLOWMYCO Sensititre™ antibiotic panel and consequently

clofazimine MIC data for *M. chimaera* are scarce. We have previously established automated quantitative drug susceptibility testing for slowly growing NTM using the MGIT 960/EpiCenter TB eXiST platform (12, 13). We here report on minimal inhibitory concentration (MIC) distributions of clofazimine and other drugs used to treat NTM infections for 48 clinical *M. chimaera* isolates and 139 other non-tuberculous mycobacteria, including 119 members of the *M. avium* complex and twenty *Mycobacterium kansasii* isolates.

MATERIALS AND METHODS

Mycobacterial strains and culture conditions. Drug susceptibility of 48 non-duplicate clinical isolates of *M. chimaera* and 139 additional slowly growing non-tuberculous mycobacteria from respiratory and non-respiratory origin including the *M. avium* complex isolates *M. avium* (n=80), *M. intracellulare* (n=31), *M. yongonense* (n=3), *M. timonense* (n=2), *M. bouchedurhonense* (n=1), *M. colombiense* (n=1) and *M. vulneris* (n=1), and *M. kansasii* (n=20) that were submitted to or isolated at our mycobacteriological laboratory from 2014 to 2018 was measured in this study (Table 1). In addition, the type strains *M. avium* ATCC 19421 and *M. chimaera* DSM 44623 were analyzed. The isolates were identified by partial 16S rRNA gene sequence analysis as described previously (14). *M. kansasii* was differentiated by sequence analysis of the *hsp65* gene (15). Mycobacteria were grown in Mycobacterium Growth Indicator Tube medium supplemented with oleic albumin dextrose catalase (OADC) (Beckton Dickinson, Sparks, MD) at 37°C.

Drug susceptibility testing. Drug susceptibility distributions of NTM were determined by automated, quantitative drug susceptibility testing (DST) using the MGIT 960 system and the Epicenter TB eXIST system (Beckton Dickinson) as described before (12, 13). The antibiotics amikacin (1, 4, 20 mg/L), clarithromycin (4, 16, 32, 64 mg/L), clofazimine (0.25, 0.5, 1, 2, 4 mg/L), ethambutol (5, 12.5, 50 mg/L), linezolid (1, 4, 16 mg/L), moxiflocaxin (0.5, 2.5, 10 mg/L), rifabutin (0.1, 0.4, 2 mg/L) and rifampin (1, 4, 20 mg/L) were analyzed. Clofazimine was purchased from Sigma (Sigma-Aldrich, Buchs, Switzerland) and dissolved in 100% dimethyl sulfoxide (DMSO). The terms susceptible (S), intermediate (I), and resistant (R) are used in this study to describe presence or absence of *in vitro* growth at a defined drug

concentration and do neither represent clinical breakpoints nor predict clinical outcome. Intermediate growth inhibition represents significant (>99%) but not complete inhibition and was categorized susceptible (S) for calculating minimal inhibitory concentration (MIC) values and depict distributions at the population level.

Clarithromycin and amikacin resistance analysis. Phenotypic clarithromycin and amikacin resistance was confirmed by sequence analysis of the 23S rRNA gene and 16S rRNA gene, respectively, as described elsewhere (16, 17). Mutations at nucleotide position A2058 and A2059 (*E. coli* equivalent) of the 23S rRNA gene were considered as resistance markers for macrolides, and mutations at nucleotide position A1408 and C1409 (*E. coli* equivalent) of the 16S rRNA gene were considered as amikacin resistance markers.

Determination of ECOFF, MIC_{50} and MIC_{90}. MIC distributions were generated from the quantitative DST results. Epidemiological cutoffs (ECOFFs) were determined by visual inspection of the MIC distributions (18). MIC_{50} and MIC_{90} was defined as drug concentration that inhibits growth of 50% and 90% of the population of a given species.

RESULTS

***M. chimaera* clofazimine MIC distribution.** Minimal inhibitory concentrations (MIC) of clofazimine were determined for 48 clinical, non-duplicate *M. chimaera* isolates using the MGIT 960/EpiCenter TB eXiST system. A clofazimine concentration range of 0.25 mg/L to 4 mg/L was tested in two-fold serial dilutions. Thirty-four out of 48 (71%) *M. chimaera* isolates were of respiratory origin, and 13 out of 48 (27%) isolates were of non-respiratory origin (Table 1). For one isolate the source was unknown. Clofazimine MIC values for *M. chimaera* ranged from ≤0.25 mg/L to 2 mg/L (Figure 1A, Table 2). MIC_{50} and MIC_{90} values of 0.5 mg/L and 1 mg/L were determined. A tentative ECOFF was set at 2 mg/L by visual inspection of the MIC distribution (Figure 1). The clofazimine MIC distribution of *M. chimaera* was compared to MIC distributions of 119 *M. avium* complex isolates including *M. avium* (n=80), *M. intracellulare* (n=31), *M. yongonense* (n=3), *M. timonense* (n=2), *M. bouchedurhonense* (n=1), *M. colombiense* (n=1) and *M. vulneris* (n=1) (Figure 1B-E, Table 2). The MIC range, MIC_{50}, MIC_{90} and tentative ECOFF of *M. chimaera* and other *M. avium* complex isolates were comparable. The clofazimine MIC distribution of *M. kansasii*, a slowly growing non-tuberculous mycobacterium not related to the *M. avium* complex, showed lower MIC_{50} (≤0.25 mg/L), MIC_{90} (0.5 mg/L) and tentative ECOFF values (1 mg/L) compared to *M. chimaera* and other *M. avium* complex species (Figure 1F).

Susceptibility distributions of additional drugs used for the treatment of *M. chimaera* infections. Susceptibility patterns of additional drugs used for the treatment of *M. chimaera* infections such as clarithromycin, ethambutol, rifampin, rifabutin, amikacin, moxifloxacin and linezolid are shown in figure 2 and table 2 for *M. chimaera, M. avium* complex species and *M. kansasii*. Susceptibility to these

drugs was in general comparable for *M. chimaera* and other members of the *M. avium* complex. Lower MIC values were observed for *M. kansasii* towards amikacin, linezolid, moxifloxacin, rifampin, and rifabutin when compared to *M. chimaera* and *M. avium* complex.

Macrolide and amikacin resistance. For two *M. avium* isolates and one isolate of each *M. chimaera* and *M. intracellulare* MIC values of ≥32 mg/L were observed for clarithromycin which indicates macrolide resistance according to CLSI guidelines (19). Sequence analysis of the 23S rRNA gene of both *M. avium* isolates and *M. intracellulare* revealed mutations at nucleotide position A2059G (*E. coli* numbering) thereby providing a genotypic confirmation of the high level macrolide resistance phenotype. However, for the *M. chimaera* isolate no mutation could be detected at nucleotide positions A2058/A2059. Repeated clarithromycin testing confirmed the decreased *in vitro* macrolide susceptibility of this isolate that was observed after prolonged macrolide treatment. Two *M. avium* and two *M. intracellulare* isolates exhibited MIC values of ≥20 mg/L for amikacin. One *M. intracellulare* isolate exhibited an A1408G mutation in the 16S rRNA gene (*E. coli* numbering) which is known to confer high-level aminoglycoside resistance (20, 21). In contrast, the second *M. intracellulare* isolate and the two *M. avium* isolates carried a wild type 16S rRNA allele. Therefore, the molecular mechanisms underlying decreased susceptibility in these strains remains elusive.

DISCUSSION

Treatment of *M. chimaera* and *M. avium* complex infections is complicated and requires multi-drug regimens. Treatment options are limited especially for macrolide-resistant isolates (7). Clofazimine, a drug traditionally used in leprosy therapy and recently recommended by the World Health Organization (WHO) for the treatment of multi-drug resistant tuberculosis (MDR-TB), is also increasingly used to treat severe *M. avium* complex infections (22, 23). Elevated MICs for clofazimine have been reported for *M. avium* and *M. intracellulare* and suggest the occurrence of resistant isolates (24). Whereas for *Mycobacterium tuberculosis* complex the WHO has released guidelines on clofazimine susceptibility testing and defined clinical breakpoints, i.e. critical concentrations, to separate resistant from susceptible isolates, such guidelines are lacking for NTM (25). Determination of MIC distributions and ECOFFs is a prerequisite for the assignment of clinical breakpoints.

Clofazimine MIC distribution data have to our knowledge not yet been reported for *M. chimaera*. Pang *et al.* reported a MIC of 0.5 mg/L for clofazimine for the type strain *M. chimaera* DSM 44623 (26). We determined the MIC_{50}, MIC_{90} and ECOFF at 0.5 mg/L, 1 mg/L and 2 mg/L, respectively, based on the MIC distribution of 48 clinical isolates of *M. chimaera* using the MGIT 960/EpiCenter TB eXiST platform and showed that these values are comparable to clofazimine MIC_{50}, MIC_{90} and ECOFF of other members of the *M. avium* complex including *M. avium* sensu stricto and *M. intracellulare*. Our data are in agreement with different reports of clofazimine susceptibility data for *M. avium* complex (24, 27-29). A MIC_{50} of 1 mg/L for *M. avium* complex was found by van Ingen *et al.* (29), and MIC_{90} values of 4 mg/L and 1 mg/L were described by Huang *et al.* for *M. avium* and *M. intracellulare*, respectively (28).

199 Luo *et al.* determined a clofazimine ECOFF of 2 mg/L for *M. avium* and
200 *M. intracellulare* (24). The clofazimine MIC distribution of *M. kansasii* was shifted
201 towards lower MICs as compared to *M. avium* complex in our study. This is in line
202 with the findings that *M. kansasii* is in general more susceptible to NTM drugs than
203 *M. avium* complex and reports of a clofazimine ECOFF of 0.5 mg/L for *M. kansasii* by
204 Luo *et al* (24).
205
206 Clofazimine resistance in NTM has been associated with mutations in TetR family
207 regulators of adjacent MmpS5-MmpL5 efflux pumps; *mmpT5* in *M. intracellulare* (30)
208 and MAB_2299c in *M. abscessus* (31). The NTM isolates characterized in this study
209 were therapy naïve regarding clofazimine, and no elevated MIC was observed.
210 Exploratory investigations in ten randomly selected *M. chimaera* isolates did not
211 reveal genetic diversity within the putative homologs RS13290 (*mmpt5;* 100% amino
212 acid (aa) sequence identity) and RS24730 (MAB_2299c; 70% aa sequence identity)
213 of *M. chimaera* DSM 44623^{T} (CP015278.1) (data not shown). In *M. tuberculosis,*
214 mutations in the Rv0678 (*mmpR5*) locus are associated with clofazimine and
215 bedaquiline resistance (32, 33). The closest homologs of Rv0678 were RS18640
216 (35% aa identity), RS15530 (35% aa identity) and RS06670 (24% aa identity) of
217 *M. chimaera* DSM 44623^{T} (data not shown). These findings confirm reports of others
218 that there is no ortholog of Rv0678 (MmpR5) in *M. avium* complex (30). Furthermore,
219 unlike RS13290 and RS24730, the latter three genes are not located in the proximity
220 of *mmpL* genes.
221
222 The MGIT 960/EpiCenter TB eXiST platform (Beckton Dickinson) is recommended
223 by the WHO for drug susceptibility testing *of M. tuberculosis,* including the testing of
224 clofazimine, and therefore available in many mycobacteria laboratories worldwide

(25). We have previously adapted MGIT 960 testing for automated quantitative drug susceptibility testing of slowly growing NTM and expanded this method for the testing of clofazimine within this study (12, 13). Commercial microdilution systems that lack clofazimine testing, e.g. the SLOWMYCO Sensititre™ panel from Trek Diagnostic Systems, are broadly used for drug susceptibility testing of slowly growing NTM (8-11). MGIT 960 testing of clofazimine, a method established in many laboratories worldwide for *M. tuberculosis* complex, could complement commercial microdilution testing for slowly growing NTM in these laboratories. Our data support the addition of clofazimine to future commercial microdilution panels for NTM.

MIC_{90} values of *M. chimaera* for drugs other than clofazimine such as amikacin, clarithromycin, ethambutol, moxifloxacin, linezolid, rifampin and rifampicin are in agreement with the findings of previous studies for *M. chimaera* and comparable to values reported for *M. avium* complex (1, 8-11, 27).

In conclusion, we provide MIC distribution, MIC_{90} and ECOFF of clofazimine for *M. chimaera* and demonstrate comparable values for other members of the *M. avium* complex. Further studies are needed to correlate *in vitro* susceptibility with clinical outcome.

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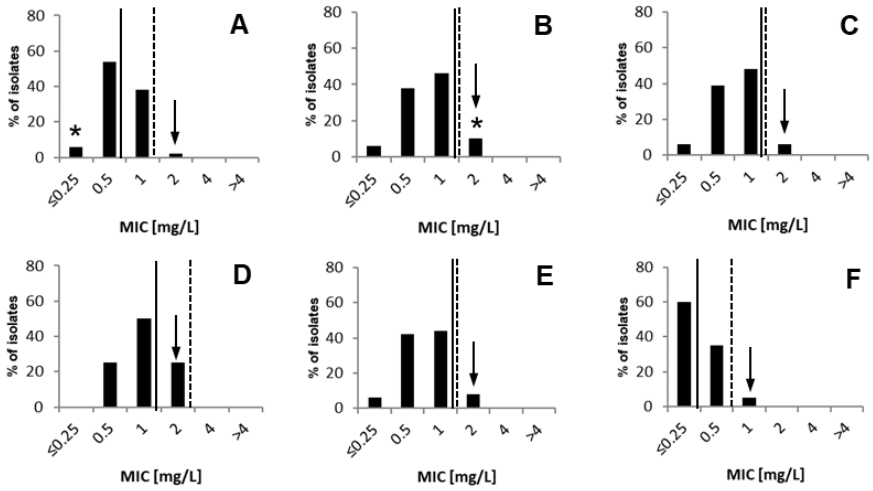
400 **FIGURES**

401

402 **FIGURE 1**



403

404 **Figure 1.** MIC distributions of clofazimine for (A) *M. chimaera* (n=48), (B) *M. avium*

405 (n=80), (C) *M. intracellulare* (n=31), (D) other MAC (n=8), (E) *M. avium* complex

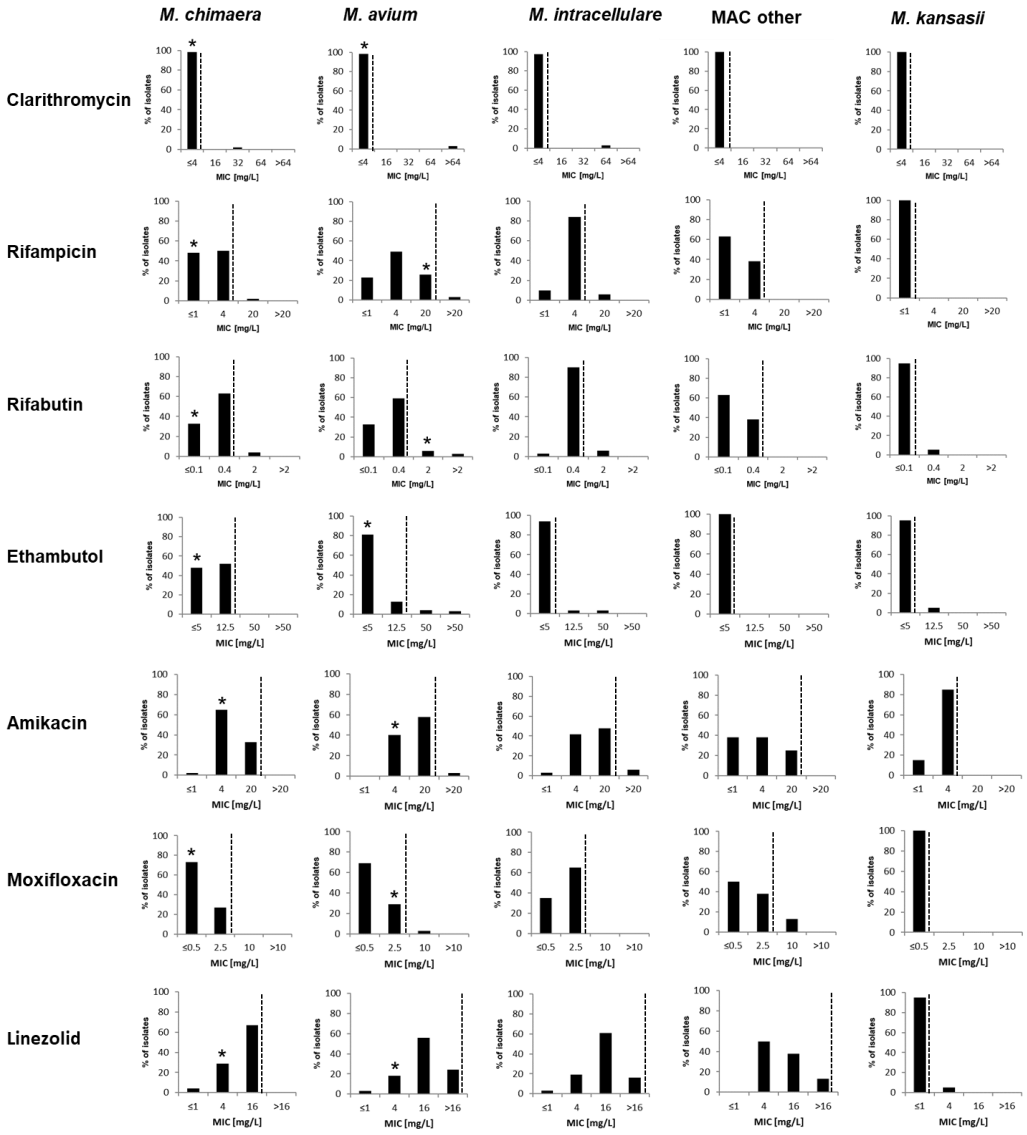
406 overall (n=167), and (F) *M. kansasii* (n=20). Tentative ECOFF (arrow), MIC_{50} (solid

407 line) and MIC_{90} (dashed line) are indicated. The clofazimine MIC value of the type

408 strains *M. avium* ATCC 19421 and *M. chimaera* DSM 44623 is indicated (*).

409

410 **FIGURE 2**



411

412

413 **Figure 2.** Susceptibility distributions for different drugs and NTM species based on

414 quantitative drug susceptibility testing data using MGIT TB eXiST. Approximated

415 MIC_{90} values are indicated (dashed line). MIC values of the type strains *M. avium*

416 ATCC 19421 and *M. chimaera* DSM 44623 are indicated (*).

417

Antimicrobial Agents and Chemotherapy

418 # TABLES

419 **Table 1. Number and origin of NTM isolates included in this study.**

Species	No. (%) of respiratory isolates	No. (%) of non-respiratory isolates	No. (%) of isolates with unknown origin	Total
M. avium (MAC)	55 (69)	8 (10)	17 (21)	**80**
M. intracellulare (MAC)	30 (97)	0 (0)	1 (3)	**31**
M. chimaera (MAC)	34 (71)	13 (27)	1 (2)	**48**
other MAC	6 (75)	1 (12.5)	1 (12.5)	**8**
M. kansasii	12 (60)	5 (25)	3 (15)	**20**

420

421

422 **Table 2. Assignment of NTM isolates to susceptibility categories in the MGIT 960**

423 **system.**

Drug/Species	MIC [mg/L]	*In vitro* DST category of isolates [n][1]			No. of isolates [n]
		S	I	R	
Clarithromycin					
M. avium	4	67	11	2	80
	16	78	0	2	
	32	78	0	2	
	64	78	0	2	
M. chimaera	4	47	0	1	48
	16	47	0	1	
	32	47	1	0	
	64	48	0	0	
M. intracellulare	4	29	1	1	31
	16	30	0	1	
	32	30	0	1	
	64	31	0	0	
other MAC	4	8	0	0	8
	16	8	0	0	
	32	8	0	0	
	64	8	0	0	
M. kansasii	4	20	0	0	20
	16	20	0	0	
	32	20	0	0	
	64	20	0	0	
Rifampicin					
M. avium	1	2	16	62	80
	4	25	32	23	
	20	60	18	2	
M. chimaera	1	9	14	25	48
	4	28	19	1	
	20	48	0	0	
M. intracellulare	1	1	2	28	31
	4	3	26	2	
	20	30	1	0	
other MAC	1	4	1	3	8
	4	5	3	0	
	20	8	0	0	
M. kansasii	1	19	0	1	20
	4	20	0	0	
	20	20	0	0	

424
425

426 **Table 2. continued.**

Drug/Species	MIC [mg/L]	*In vitro* DST category of isolates [n][1]			No. of isolates [n]
		S	I	R	
Rifabutin					
M. avium	0.1	11	15	54	80
	0.4	39	34	7	
	2	78	0	2	
M. chimaera	0.1	9	7	32	48
	0.4	36	10	2	
	2	47	1	0	
M. intracellulare	0.1	0	1	30	31
	0.4	10	19	2	
	2	31	0	0	
other MAC	0.1	3	2	3	8
	0.4	5	3	0	
	2	8	0	0	
M. kansasii	0.1	20	0	0	20
	0.4	20	0	0	
	2	20	0	0	
Ethambutol					
M. avium	5	46	19	15	80
	12.5	71	4	5	
	50	78	0	2	
M. chimaera	5	19	4	25	48
	12.5	47	1	0	
	50	48	0	0	
M. intracellulare	5	27	2	2	31
	12.5	30	0	1	
	50	31	0	0	
other MAC	5	7	1	0	8
	12.5	8	0	0	
	50	8	0	0	
M. kansasii	5	19	0	1	20
	12.5	20	0	0	
	50	20	0	0	

427
428

429 **Table 2. continued.**

Drug/Species	MIC [mg/L]	*In vitro* DST category of isolates [n][1]			No. of isolates [n]
		S	I	R	
Amikacin					
M. avium	1	0	0	80	80
	4	5	27	48	
	20	72	6	2	
M. chimaera	1	0	1	47	48
	4	18	14	16	
	20	48	0	0	
M. intracellulare	1	1	0	30	31
	4	3	11	17	
	20	28	1	2	
other MAC	1	3	0	5	8
	4	4	2	2	
	20	8	0	0	
M. kansasii	1	3	0	17	20
	4	20	0	0	
	20	20	0	0	
Moxifloxacin					
M. avium	0.5	29	26	25	80
	2.5	75	3	2	
	10	79	1	0	
M. chimaera	0.5	11	24	13	48
	2.5	48	0	0	
	10	48	0	0	
M. intracellulare	0.5	3	8	20	31
	2.5	30	1	0	
	10	31	0	0	
other MAC	0.5	3	1	4	8
	2.5	7	0	1	
	10	8	0	0	
M. kansasii	0.5	20	0	0	20
	2.5	20	0	0	
	10	20	0	0	

430
431

432 **Table 2. continued.**

Drug/Species	MIC [mg/L]	*In vitro* DST category of isolates [n][1]			No. of isolates [n]
		S	I	R	
Linezolid					
M. avium	1	1	1	78	80
	4	8	8	64	
	16	27	34	19	
M. chimaera	1	0	2	46	48
	4	3	13	32	
	16	40	8	0	
M. intracellulare	1	0	1	30	31
	4	2	5	24	
	16	12	14	5	
other MAC	1	0	0	8	8
	4	1	3	4	
	16	5	2	1	
M. kansasii	1	15	4	1	20
	4	20	0	0	
	16	20	0	0	
Clofazimine					
M. avium	0.25	1	4	75	80
	0.5	9	26	45	
	1	44	28	8	
	2	80	0	0	
	4	80	0	0	
M. chimaera	0.25	3	0	45	48
	0.5	24	5	19	
	1	39	8	1	
	2	48	0	0	
	4	48	0	0	
M. intracellulare	0.25	0	2	29	31
	0.5	2	12	17	
	1	17	12	2	
	2	30	1	0	
	4	31	0	0	
other MAC	0.25	0	0	8	8
	0.5	1	1	6	
	1	4	2	2	
	2	8	0	0	
	4	8	0	0	
M. kansasii	0.25	9	3	8	20
	0.5	18	1	1	
	1	20	0	0	
	2	20	0	0	
	4	20	0	0	

[1] The categories susceptible (S), intermediate (I), and resistant (R) are used in this study to describe presence or absence of *in vitro* growth at a defined drug concentration and do neither represent clinical breakpoints nor predict clinical outcome. Intermediate growth inhibition represents significant (>99%) but not complete inhibition and was categorized susceptible (S) for calculating minimal inhibitory concentration (MIC) values and depicting distributions at the population level.